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Response of Grey Poplar (*Populus* x *canescens*) to Copper Stress

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ABSTRACT

Copper (Cu) is an essential element for plant growth and development, but it can be toxic when available in excessive amounts. This study aimed to determine Cu sensitivity (0-500 μM) in stem height and radial growth in relation to photosynthetic performance and stress enzymes in young *Populus* x *canescens* trees. Biomass and leaf formation rate were unaffected by Cu variation in the range from 0.128 μM (normal supply with the nutrient solution) to 5 μM. Higher Cu concentrations caused reductions in all growth parameters and severe leaf injury. The quantum yield of photosystem II was decreased at Cu concentrations above 5 μM, but recovered in darkness almost completely indicating high Cu tolerance of photosystem II despite foliar damage. The activities of stress enzymes such as guaiacol peroxidase, glutathione peroxidase and NADH oxidase showed no increase with growth reductions suggesting that H_2O_2 was not involved in stress symptoms. Cu deficiency stimulated root growth. Modulation of Cu supply in the optimum range affected the relation between elongation and radial growth indicating differences in the Cu demand of these processes.

Keywords: growth, biomass, heavy metal, peroxidase, photosynthesis, tolerance
Abbreviations: BCA, bicinchoninic acid; **BSA**, bovine serum albumin; **GSH**, glutathione; **NADPH**, nicotinamide adenine dinucleotide phosphate; **PAR**, photosynthetically active radiation; **POD**, peroxidase; **PS II**, photosystem 2

INTRODUCTION

A serious environmental problem is the accumulation of heavy metals in plants as a result of pollution of soils and water. Anthropogenic factors such as industrial activities of men, mining, sewage disposal, traffic, smelting, electroplating and ore refining have resulted in increasing contamination of soil, water and air (Schützendübel and Polle 2002). Further, acid rain increases soil acidity. Lowering of soil pH mobilizes and drives leaching of nutrient cations and increases the availability of toxic heavy metals. Such changes in the soil chemical characteristics reduce soil fertility, which ultimately results in negative impact on growth and productivity of trees and crop plants (Singh and Agrawal 2008).

Among the heavy metals that are accumulating in natural ecosystems, the focus of the present work is on Cu. Cu, is a redox-active transition metal and an essential micro-nutrient for plants (Marschner 1995). But, it can also be a toxic element when tissue concentrations exceed the optimal demand (Ducic and Polle 2005; Yruela 2005). Most of the functions of Cu as a plant nutrient are based on the participation of enzymatically-bound Cu in redox reactions. Many studies showed that at the biochemical level, Cu toxicity induced stress enzymes involved in the detoxification of reactive oxygen species (Ducic and Polle 2005).

A stimulating effect of Cu on plant growth was noted earlier when using Cu salts as fungicides (Sommer 1931) indicating that too low Cu supply can also have negative effects. However, continued application of such fungicides caused accumulation of Cu in agricultural soils threatening environmental quality and reducing soil fertility for crop growth (Strawn and Baker 2008). It is well known that variations of Cu outside the sufficient or optimum range influence plant performance. For example, in poplar (*P. trichocarpa* x *P. deltoids* hybrid) Cu deficiency affected root growth more than shoot growth (Van Den Driessche 2000). In *P.* x *canescens*, low concentrations of Cu did not cause chlorosis, or browning and did not suppress shoot development (Bojarczuk 2004). However, high concentrations inhibited shoot and root development.

It has been suggested to use poplars for phytoremediation (Lasat 2002; Gratão *et al*. 2005; Peuke and Rennenberg 2005; Ghost and Singh 2005) as well as for monitoring of heavy metal pollution of air and soils (Sawidis *et al*. 1995; Madejon *et al*. 2004; Suleyman *et al*. 2005; Berlizov *et al*. 2007). Woody species with high biomass production, deep root system, high growth rate, high capacity to grow in impoverished soils, and high capacity to allocate metals to the trunk, may be especially suitable in this respect (Almeida *et al*. 2007). This applies particularly to *Populus* species because of their fast growth rate (Taylor 2002; Tsakou *et al*. 2003), extensive root system (Koprivova *et al*. 2002) and high biomass production (Laureysens *et al*. 2004). A recent study of Borghi *et al*. (2008) on two poplar species (*P*. x *canadensis* and *P. alba*) showed different responses to high Cu concentrations. The authors detected high Cu accumulation in roots of *P*. x *canadensis* suggesting its suitability for phytostabilization, whereas *P. alba* accumulated Cu in leaves indicating Cu polluted soils.

To date, little is known about the Cu requirements and sensitivity of trees. The main aim of this experiment was to determine the Cu sensitivity of height and stem growth in hybrid poplar (*P*. x *canescens*) and relate plant performance to photosynthesis and biochemical stress responses.

MATERIALS AND METHODS

Production of plants, growth conditions and Cu exposure

Grey poplar *P*. x *canescens*, a hybrid of *P. tremula* x *P. alba* plants were produced by *in vitro* micro-propagation after the method of Leplé *et al*. (1992). Micro-cuttings were prepared in a rooting medium (modified after Murashige and Skoog 1962) under sterile conditions. Rooted plantlets were placed into hydroponic nutrient solution (modified Long Ashton medium (Hewitt and Smith 1975) in a culture room under controlled conditions (air temperature 22 ±

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1°C, photoperiod 18 h with 184 ± 7 µmol quanta m^{-2} s^{-1} photosynthetically active radiation, PAR and a relative air humidity of 60 ± 5%) for three weeks. The nutrient solution was changed regularly once a week. Subsequently, the plants were transferred to a greenhouse equipped with supplementary lighting of approximately 180-200 µmol quanta m^{-2} s^{-1} PAR (16 h photoperiod, F58 W/125 T8 fluorescent lamps, Havells Sylvania GmbH, Erlangen, Germany). The temperature fluctuated between 21 and 25°C.

Based on uniformity in shoot height (mean height 17.1 ± 2.6 cm), 36 plants were selected. Six plants were placed in a box with 20 l of nutrient solution. Each box was used for one of the following Cu treatments: 0, 0.128, 1, 5, 50, and 500 µM of Cu (supplied as $CuSO_4 \cdot 5H_2O$). The nutrient solutions were constantly aerated with sterile air and exchanged regularly once a week. The plants were exposed to the range of different Cu concentrations for two weeks.

Chlorophyll fluorescence

Chlorophyll fluorescence was determined by using a pulse modulated chlorophyll fluorometer (MINI-PAM, Walz, Effeltrich, Germany) on the upper surface of the first fully-expanded leaf at the top of each plant. The yield of chlorophyll fluorescence was determined in light (with approximately 180-200 µmol quanta m^{-2} s^{-1} PAR) and in darkness (before the light started).

The quantum yield of photosystem II (Φ) was calculated according to Genty *et al*. (1989):

$\Phi_{dark} = (F_m - F_0)/ F_m$ and $\Phi_{light} = (F'_m - F'_0)/F'_m$

where Φ_{dark} = maximum quantum yield of photosystem II, Φ_{light} = actual quantum yield of photosystem II, F_m = maximum fluorescence in darkness, F'_m = maximum fluorescence in light, F'_0 = basic fluorescence in light, and F_0 = basic fluorescence in darkness.

Growth and biomass

To monitor growth, height of the plant shoot, stem diameter at the root neck and leaf numbers were determined regularly once a week. The growth rate during Cu exposure was calculated in the last week of Cu treatment. After 14 days of Cu exposure the plants were harvested. Each plant was separated into shoot and root. The shoot was divided into leaves and stem. The root was divided into fine and coarse roots. The fresh mass of each plant fraction was determined. Two to three fully expanded leaves from each plant were removed, frozen in liquid nitrogen and stored at –80°C for biochemical analysis. All residual plant materials were dried at 70°C for seven days and used for dry mass determination.

Biochemical analysis

Total leaves of each plant were pooled at harvest and two to three leaves were used for the biochemical analysis. Frozen leaves (1 g) were ground in liquid nitrogen, extracted in buffer and gel-filtered as described previously (Schützendübel *et al*. 2001) and used to determine the activities of guaiacol peroxidase (EC 1.11.1.7) after Polle *et al*. (1990), NADPH oxidase (EC 1.6.99.3) after Polle *et al*. (1992) and glutathione peroxidase (EC 1.11.1.9) according to Drotar *et al*. (1985). The total soluble protein content was determined with bicinchoninic acid method, using BSA (2 mg/ml) as the standard according to the instructions of the manufacturer (Uptima, Montflucon, France). For each replicate, 30 µl from the standard protein (0, 25, 50, 75, 100, and 200 µg/ml) or from the sample was mixed with the bicine choninic reagent in a 1.5 reaction tube (Eppendorf, Sarstedt, Nümbrecht, Germany). The reaction of the mixture was enhanced by incubating the tubes in a water bath at 60°C for 30 min. After incubation, the samples and standards were placed on ice for 1 to 2 min to cool down. The extinction was measured by a spectrophotometer (UV-DU® 640, Beckman Instruments Inc., Fullerton, USA) at 25°C and the wavelength of 562 nm.

Statistical analysis

Data were analysed with the statistical programme JMP 5.1 Start Statistics, 3rd edition (SAS Institute, Inc., Cary, North Carolina, U.S.A) using One-Way-ANOVA. Data are the mean of six replicates per Cu treatment. The separation of the means was performed by Tukey's test. A probability level for $P \leq 0.05$ was considered to indicate significant differences.

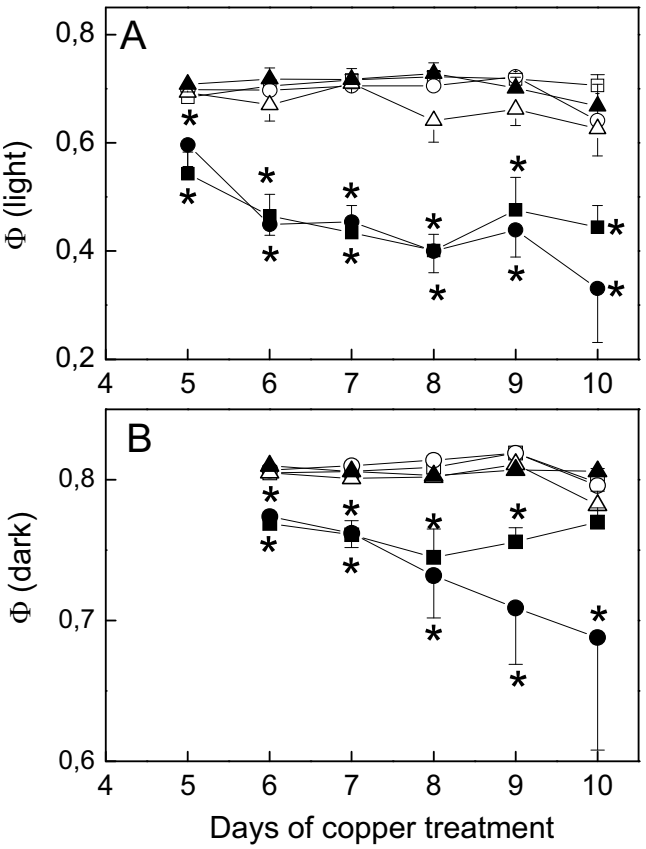

Fig. 1 Effect of increasing Cu concentrations on the quantum yield of photosystem II (Φ) of *Populus* x *canescens* in light (**A**) and in darkness (**B**). The following Cu concentrations were applied: 0 (open square), 0.128 (open circle, control), 1 (open triangle), 5 (closed triangle), 50 (closed square) and 500 µM Cu (closed circle). Data are means (n = 6 plants for each Cu treatment, ± SE). Stars indicate significant differences at $P \leq 0.05$ from controls.

RESULTS

Chlorophyll fluorescence

To examine the effect of different Cu treatments on the photosynthetic performance of *P.* x *canescens*, the yield of chlorophyll fluorescence (Φ) was determined in light and darkness. Cu concentrations of 50 and 500 µM caused significant reductions in the photosynthetic yield in light, which were initially almost fully reversible in darkness (**Fig. 1A, 1B**). With progressing exposure time, older leaves were shed and younger leaves showed severe injury such as drying. After two weeks of Cu exposure, the reduction of the actual quantum yield of photosystem II was more than 50% with the highest Cu treatment. Still, the maximum reduction in photosynthetic yield in darkness was only 10% compared with controls (**Fig. 1B**). This shows that excessive Cu caused only moderate injury to photosystem II and mainly resulted in down-regulation of electron transport.

Growth performance

To analyse the performance of *P.* x *canescens* plants exposed for two weeks to different Cu treatments, the growth was monitored by measuring shoot height, leaf numbers and stem diameter at the root neck regularly.

The leaf formation rate was significantly reduced in plants that received the highest Cu concentrations of 50 and

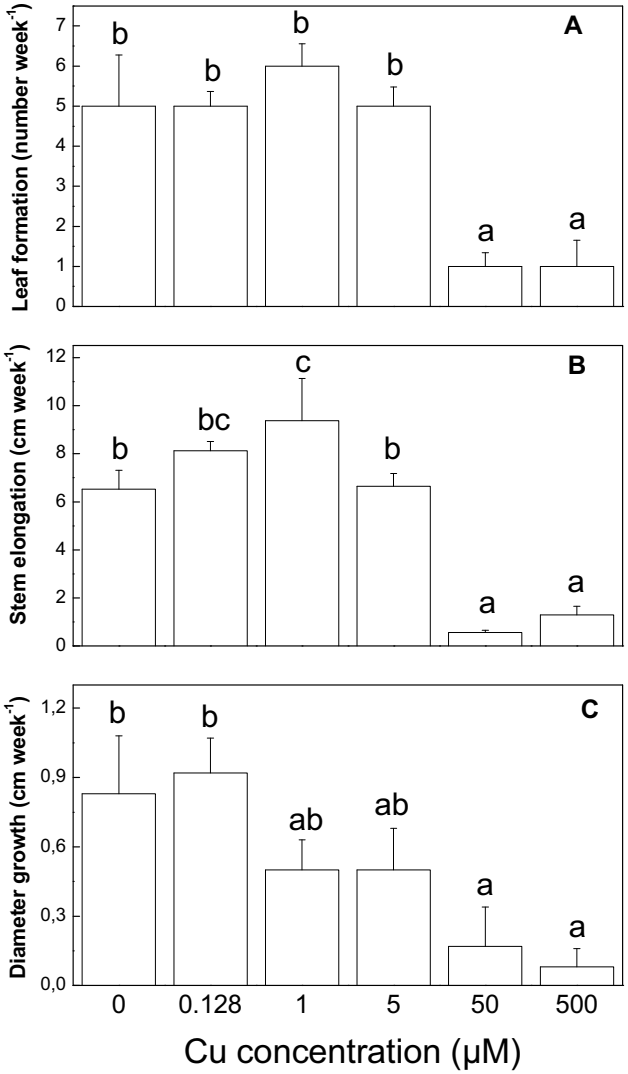

Fig. 2 Influence of copper on leaf formation (**A**), height growth (**B**) and stem diameter growth (**C**) of *Populus* x *canescens*. Cu treatments of 0, 0.128, 1, 5, 50 and 500 µM Cu lasted for two weeks. Growth measurements were performed on three occasions following the application of Cu treatments. Data are mean growth rate of six plants per treatment (n = 6, ± SE). Different letters indicate significant differences at $P \leq 0.05$.

500 µM Cu, respectively, compared with control plants (**Fig. 2A**). Plants supplied with 1 µM Cu in the nutrient solution showed maximum stem height growth (**Fig. 2B**), whereas moderate decreases in stem elongation were observed in plants supplied with less (0.128 µM Cu) or no Cu as well as in plants exposed to 5 µM Cu. Plants exposed to 50 or 500 µM Cu showed no stem elongation any more (**Fig. 2B**). In contrast to stem elongation, maximum radial growth was found in presence of 0.128 µM Cu, which is the normal concentration of standard nutrient medium. Cu depletion for two weeks did not affect radial growth. Radial growth was more sensitive to higher Cu concentrations than elongation growth since reductions were already found at 1 µM Cu in the nutrient solution.

Biomass

After two weeks of exposure to different Cu treatments, the dry mass of *P.* x *canescens* was determined. Significant reductions in total plant dry mass were only found in plants treated with 50 or 500 µM Cu, whereas all other treatments had only marginal effects compared with the controls receiving 0.128 µM Cu in the nutrient solution (**Table 1**). No significant variations in the root/shoot ratio were detected in response to increasing Cu supply (**Table 1**). However, in the absence of Cu the root-to-shoot ratio increased, pointing to a relative stimulation in root formation (**Table 1**).

Table 1 Effects of different Cu treatments on dry mass and root/shoot ratio of *Populus* x *canescens*. Plants were analysed after 2 weeks of Cu exposure. Data are means (n = 6, ± SE). Different letters indicate significant differences at $P \leq 0.05$.

Cu treatment (µM)	Total plant DW (g)	Root-to-shoot ratio
0	4.3 ± 0.9 abc	0.21 ± 0.02 b
0.128	6.0 ± 0.4 c	0.14 ± 0.01 a
1	5.1 ± 0.5 abc	0.14 ± 0.02 a
5	5.3 ± 0.8 bc	0.15 ± 0.01 a
50	2.7 ± 0.5 a	0.16 ± 0.01 a
500	3.1 ± 0.3 ab	0.14 ± 0.01 a

Biochemical analysis

To find out whether excess Cu stimulated the defence system, total soluble protein content and the activities of peroxidases with glutathione or guaiacol as substrates and of NADH oxidase were determined in leaves. The total soluble protein content and the activities of glutathione POD showed no variation between the control plants and any of the other Cu treatments (**Table 2**). In contrast, the activities of NADH oxidase were significantly higher in the controls than in any of the other Cu treatments. Guaiacol POD activities decreased with increasing Cu concentration leading to significant effects in the presence of 50 µM Cu (**Table 2**). Leaves of plants exposed to 500 µM Cu showed severe injury and drying symptoms; they were not analysed.

DISCUSSION

In this study, Cu sensitivity of *P.* x *canescens* growth performance was investigated in relation to photosynthesis and biochemical stress responses. The Cu concentrations applied here were in a range similar to those employed in other studies, where poplars were exposed up to 1000 µM Cu (Bojarczuk 2004; Borghi *et al.* 2007). Under field conditions, soil Cu concentrations can also vary over a wide range (Ducic and Polle 2005). On contaminated sites up to 990 mg Cu kg^{-1} have been found (Yoon *et al*. 2006). However, it is important to note that the plant availability of elements in nutrient solutions is usually much higher than in soil because of exchange processes between soil solution and soil particles. Experiments in hydroponics, therefore, provide conservative estimates of plant sensitivity to nutrient toxicity.

In accordance with previous studies, grey poplar showed severe growth inhibition and injury when exposed to Cu concentrations above 50 µM (Bojarczuk 2004; Borghi *et al.* 2007). Other tree species such as *Pinus pinea*, *Pinus pinaster*, *Fraxinus angustifolia* and *Prunus cerasifera* were more sensitive to excess Cu than poplar because growth reduction occurred at concentrations from 5 to 16 µM (Arduini *et al.* 1995, 1996; Lombardi and Sebastiani 2005). Similarly, Wojcik and Tukiendorf (2003) showed that hydroponically-grown *Arabidopsis thaliana* treated with 5 to 100 µM Cu for two weeks exhibited a progressive decrease of root length and biomass with increasing Cu in the nutrient solution. Overall data from hydroponic experiments indicated that heavy metals affect root cell elongation and decrease mitotic activity with a consequent inhibition of root growth (Polle and Schützendübel 2003). Our study showed that excess Cu inhibited shoot and root biomass formation to a similar extent as the root-to-shoot ratio was unaffected. A relative increase in root biomass occurred, however, in response to Cu deficiency, possibly indicating an attempt of the plant to increase "soil" exploration for this limiting element.

The photosynthetic apparatus of poplar is obviously relatively stable against injury imposed by Cu as we found mainly down-regulation of electron transport and only little

Table 2 Effects of Cu treatments on total soluble protein content and the activities of guaiacol peroxidase, glutathione peroxidase and NADH oxidase of *Populus* x *canescens*. The measurements were performed after 2 weeks of Cu exposure in leaf extracts. Data are means (n = 6, ± SE). Different letters indicate significant differences at $P \leq 0.05$.

Cu treatment (µM)	Soluble protein (mg g^{-1} FW)	Guaiacol POD (nkat g^{-1} FW)	GSH POD (nkat g^{-1} FW)	NADH-oxidase (nkat g^{-1} FW)
0	33.7 ± 2.0 a	591 ± 43 a	2.4 ± 0.9 a	3.4 ± 0.7 b
0.128	39.8 ± 4.5 a	580 ± 64 a	3.4 ± 0.2 a	14.8 ± 3.6 a
1	32.8 ± 2.0 a	471 ± 26 ab	4.0 ± 0.2 a	3.8 ± 0.9 b
5	36.6 ± 2.3 a	473 ± 38 ab	3.2 ± 0.3 a	4.8 ± 1.0 b
50	39.4 ± 3.2 a	352 ± 94 b	3.5 ± 0.9 a	6.9 ± 2.5 b

injury of PS II. As PSII injury was low even in plants with severe leaf injury, it is concluded that fluorescence yield is an inadequate indicator for Cu stress. Growth of *P.* x *canescens* is apparently more sensitive than photosynthesis. This suggestion is also supported by a recent study of Kovacs *et al*. (2005), who compared the effects of Cu and Cd on leaf development in poplar. They demonstrated that growth was more strongly reduced by Cu treatment, while Cd preferably inhibited photosynthesis. Strategies to improve heavy metal tolerance by transgenic modifications as in Koprivova *et al*. (2002) and Gyulai *et al*. (2005), therefore, must be specifically developed for different metal stresses. To improve Cu tolerance they should be targeted at improving protection of other compartments than chloroplasts.

In our study cell biochemistry, such as NADH oxidase activities and protein content were little affected by Cu stress. In contrast to several other species (Radotic *et al*. 2000; Wang *et al*. 2004; Uzunova *et al*. 2004; Gao *et al*. 2008), poplar leaves showed decreases in guaiacol peroxidase activities when exposed to excess Cu. The inactivation of peroxidases seems to be typical for poplar since suppression of peroxidases was also found in roots of mature trees of black poplar (*Populus nigra*) and cottonwood (*Populus deltoides*) in a heavy metal polluted environment (Stobrawa and Lorenc-Plucinska 2007).

Among the parameters analysed, stem radial increment was the most Cu-sensitive growth process compared with shoot elongation or leaf formation. As our experiment lasted only for two weeks, this inhibition did not yet cause loss in stem biomass production. However, this may be expected under long term conditions. For example, exposure of Scots pine to Cu and Ni pollution resulted in retardation in tree diameter growth over 15 years after contamination of the field site (Fedorkov 2007). Our study demonstrates a remarkable sensitivity of radial growth in poplar, which occurred at concentrations as low as 1 µM Cu in the nutrient solution, whereas photosynthetic electron transport was only injured at 50 µM Cu. Future studies must elucidate why elongation growth is less Cu-sensitive than radial growth. Further analysis of the underlying reasons is required if poplars are to be introduced for phytoremediation because the most sensitive factor will determine the suitability of this species for soil clean up.

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